Genetic and Molecular Dosimetry of HZE Radiation

Gregory A. Nelson, Wayne W. Schubert, Gayane A. Kazarians & Gil F. Richards
Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91109 USA

Eugene V. Benton, Eric R. Benton & Richard P. Henke
University of San Francisco, San Francisco, California 94117 USA

Introduction

One of the unavoidable risks of spaceflight is exposure to the complex space radiation environment which can result in a 50-fold increase in exposure relative to life at the surface of the earth. Many different forms of radiation contribute to this exposure including electromagnetic radiation (X-rays, gamma rays, ultraviolet light), neutrons and charged particles such as protons, alpha particles and nuclei of other elements The charged particles, often referred to as HZE particles or cosmic rays, represent a unique space hazard due to their penetrating nature and the fact that they deposit their energy in highly structured tracks. Such tracks concentrate damage to columns of cells on a micron scale even though the macroscopic dose on a centimeter scale is considered small.

Radiation exposure can lead to pathological changes in living cells and tissues. Of greatest importance are changes to chromosomes which ultimately lead to conversion of normal cells to cancer ceils. Loss of function in cells of the lens resulting in cataracts or alterations in germ cells which may lead to heritable defects arc also of medical importance. The physiological changes that result from exposure to microgravity may modify the cell or body's ability to manage or repair damage induced by radiation, thus introducing an additional uncertainty in the evaluation of health risks to astronauts and cosmonauts who live and work in space.

This experiment attempts to isolate genetic changes in animal cells caused by cosmic rays in space so that their likelihood of occurrence and structural features can be evaluated. The space measurements are evaluated against results from synthetic cosmic rays produced by particle accelerators on the ground. We used the tiny soil nematode, *Caenorhabditis elegans*, as a model animal for these studies and developed special correlative methods for identifying mutations caused by high energy charged particles present at relatively low abundance on the IML-1 mission (Figure 1).

Materials and Methods

The nematode, *Caenorhabditis elegans*, var. Bristol is a free-living soil nematode whose development and genetics have been the subject of intensive investigation[9]. The animal exists as a self-fertilizing hermaphrodite or as a male depending upon the number of X chromosomes present. It has six pairs of chromosomes and a genome whose size approximates one average human chromosome. C. *elegans* has a fixed number of cells and a highly reproducible life cycle which is temperature dependent. At 20 degrees C an egg will hatch, develop through four larval stages punctuated by molts, complete spermatogenesis, switch to oogenesis and produce its first offspring by self-fertilization in three days. An animal will produce approximately 280 "self-cross" offspring in the next 3 days and will survive to an old age of about 3 weeks. If mated by a male, male sperm will outcompete endogenous sperm for eggs and a hermaphrodite may produce up to 1000 "outcross" offspring in a 1:1 sex ratio. Ordinarily, males arise al a frequency of 1/700 by controlled X-chromosome disjunction. Under

Fig. 1. A late larval hermaphrodite with two early larvae grazing on a lawn of *E. coli* bacteria. The animals are offspring of second generation worms which developed in microgravity. The frame size is 1 mm wide,

conditions of crowding or environmental stress worms may enter an estimating state called the dauer larva. In this state the animals do not feed and maybe kept for several months as long as they are oxygenated and hydrated. Upon return to a food supply, dauer larvae resume development at the third larval molt. The two main objectives of the experiment required life support conditions for worms in both growing populations and as dauer larvae.

Two hardware configurations were used to house nematodes in the Biorack and Spacelab. For dormant populations of dauer larvae, and growing populations of worms, a simple culture tube was used in Type I containers as shown in Figure 2. Ten thousand N2 (wild type) or JP 10 tester-strain dauer larvae were placed in one milliliter of standard phosphate buffered saline and loaded into a tube with approximate y 2 milliliters of air space. For growing populations a thin cylindrical shell of agarose medium was poured into tubes using a removable glass rod. The thin film cultures were inoculated with *E. coli K12* strain *X1666* kept growth arrested with antibiotics: kanamycin, streptomycin and nystatin. The surface areas of the thin film cultures are roughly equivalent to a standard 35mm petr dish culture. Six or twelve hermaphrodite and male animals of genetically marked strains were inoculated prior to launch and allowed to reproduce during the flight. In order to characterize the radiation environment on a scale of about 1 cm, thermoluminescent detectors and CR-39 plastic nuclear track detectors were packaged with 4 or 8 tubes in each Type I container. Thermoluminescent detectors

Type I Container Internal Contents

Fig. 2. **Growing cultures of worms were placed in lexan tubes seeded with a thin layer of agarose and *E. coli* bacteria. Dormant dauer larvae were incubated in 1 milliliter cultures of buffer. In order to define the radiation field in each container thermoluminescent detectors (TLD) and CR-39 plastic nuclear track detectors were placed adjacent to tubes. Type I containers were dated in the Biorack at 22 and 4 degrees at O-gravity and on the l-g centrifuge. Additional samples were placed in the Spacelab tunnel where radiation shielding was at a minimum.**

Fig. 3. In order to correlate individual cosmic rays with "hit" worm larvae, a laminated "sandwich" of particle detectors, support layers, and nematode immobilization layers was used. Ray tracing techniques allowed particle tracks and corresponding worm locations to be identified.
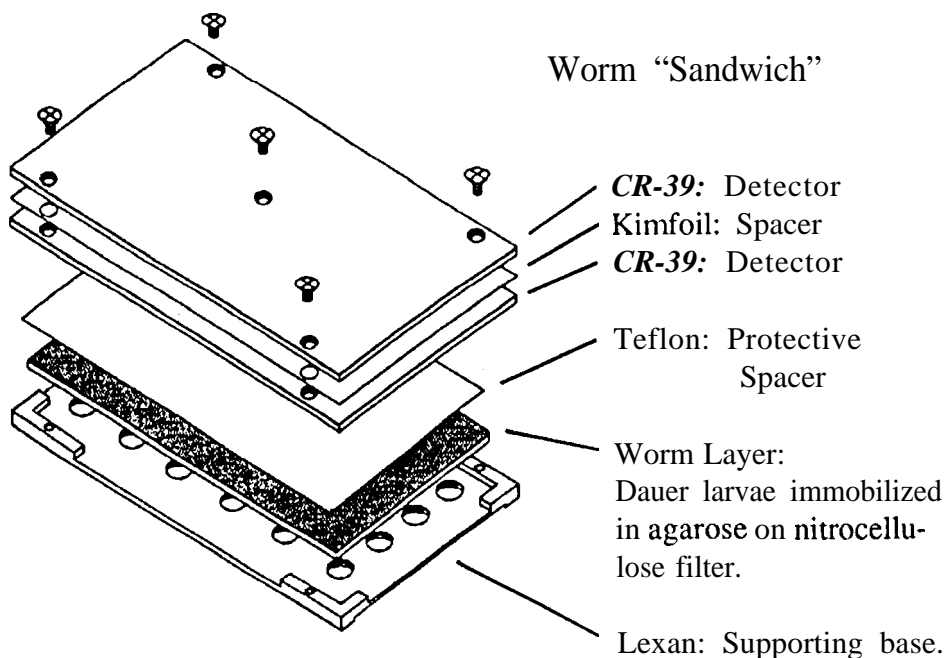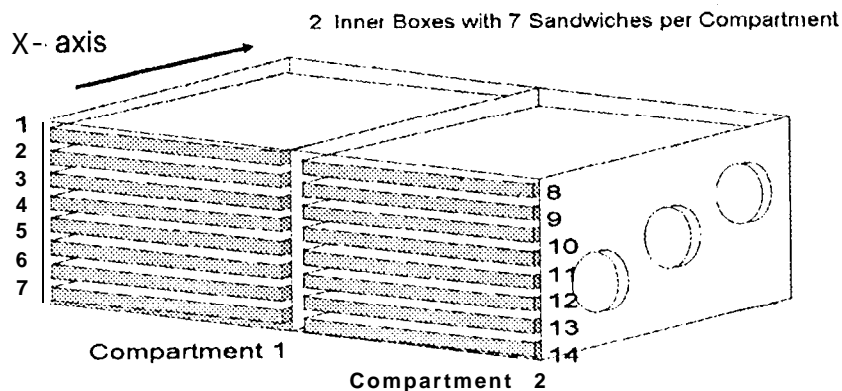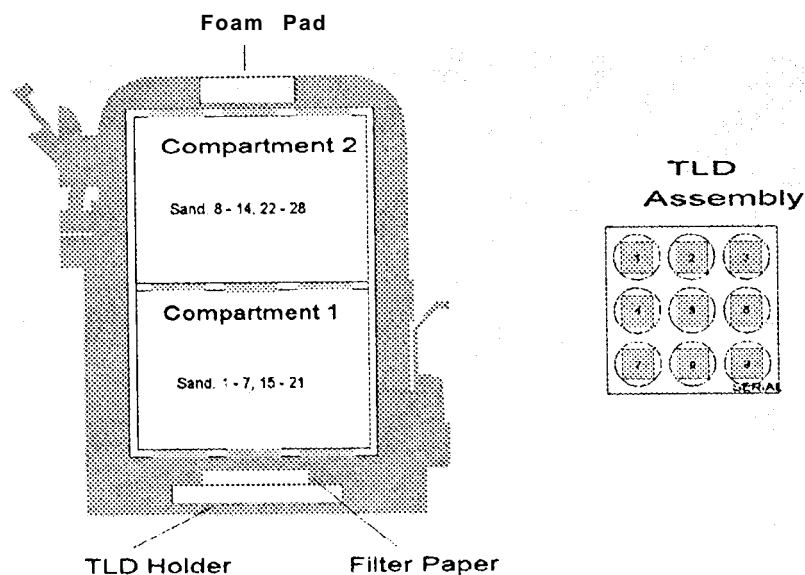
Worm "Sandwich"

CR-39: Detector
Kimfoil: Spacer
CR-39: Detector

Teflon: Protective
      Spacer

Worm Layer:
Dauer larvae immobilized
in agarose on nitrocellu-
lose filter.

Lexan: Supporting base.

Fig. 4. Nematode" Sandwich" assemblies were packaged in ventilated retaining boxes and placed in Type II containers modified with air holes covered with thin silicone membranes to prevent moisture loss and contamination. Thermoluminescent detectors were included to measure total radiation dose. The two Type 11 containers were incubated at 4 degrees C in order to effectively immobilize the worms.

Type II Internal Hardware

Foam Pad

Compartment 2

Sand. 8 - 14, 22 - 28

Compartment 1

Sand. 1 - 7, 15 - 21

TLD
Assembly

TLD Holder   Filter Paper

X - axis

2 Inner Boxes with 7 Sandwiches per Compartment

1
2
3
4
5
6
7

8
9
10
11
12
13
14

Compartment 1

Compartment 2

integrate the total dose from all forms of ionizing radiation whereas CR-39 detects , tracks from charged particles and allows spectral information to be calculated. Three dimensional information on charged particle fluence was derived from CR-39 stacks in four-tube Type 1's. Radiobiological measurements from Type I samples is therefore correlated with average radiation environments.

In order to measure genetic changes associated with specific charged particles a second hardware configuration was used. Dauer larvae were immobilized onto a layer of nitrocellulose filter at approximately 10,000 worms per square centimeter by overlaying them with 4% agarose at 4 degrees C. Under these conditions the worms can be held in place for about 1 month. The filter was then placed on a plastic support layer with aeration holes and assembled with two parallel layers of CR-39 spaced by a lexan foil and a protective Teflon layer as shown in Figure 3. The "sandwich" can be disassembled for processing of the CR-39 and analysis of etch-cone tracks by a computer-aided videomicroscopy which identifies particles and their points of penetration in the worm layer. 0.5 mm diameter plugs containing "hit" dauer larvae were then extracted for genetic analysis. 28 "sandwiches" were packaged into two Type 11 containers and incubated at 4-5 degrees C for the duration of the mission. As with Type I containers, a TLD package was included to provide total dose. The internal configuration of a Type 11 container is shown in Figure 4.

Development and Chromosome Mechanics in Microgravity

Strains used for development studies were obtained from the *Caenorhabditis* Genetics Center and contained the following recessive alleles: *dpy-5(e61)I*, *unc-13(e51)I*, *dpy-17(e164)III*, *dpy-18(e364)III*, *unc-32(e189)III*, *unc-5(e53)IV*, *unc-22(bc96)IV*, *dpy-11 (e224)V, and him-5(e1490)V. The* phenot ypes of *dpy* mutants are **dump y,** abnormall y short and of approximate] y normal diameter. *Unc* uncoordinated mutants have defects resulting in partial to complete paralysis or rhythmic "twitching" (uric-22). *Him* high incidence of male mutants are defective in X-chromosome disjunction and produce up to 4090 male offspring.

Four tests were earned out to assess development and the behavior of chromosomes during meiosis. Each test was performed with two different genotypes to control for specific gene effects. These tests arc referred to as: "growth", "mating", "segregation" (of unlinked markers) and "recombination" (plus segregation of linked markers). Worms from selected cultures were fixed and stained using the fluorescent DNA-binding dyc DAPI (diamidinophenolindole) to reveal cell nuclei. Some embryos rccovercd at landing were also fixed and stained with antibodies specific for cytoplasmic determinants that localize to different cell lineages. In every case simultaneous ground controls were performed using sibling animals or identical subcultures in the duplicate Biorack run at NASA's Hanger L near the launch site. Tubes were unloaded for processing at Dryden Test Flight Facility about 6 hours after de-orbit bum corresponding to mid embryogenesis.

Growth

Six third larval stage (L3) Wild Type or mutant genotype animals were inoculated into tubes incubated in the Spacelab tunnel belt or at 22 degrees C in Biorack incubator A and allowed to grow and rcproducc. After recovery popula- . t ion distribut ions of worms were measured, sets of animals were fixed or frozen for DAPI anti antibody staining. Embryos were also isolated by dissection of gravid hermaphrodites and preserved for antibody staining.

Vigorous growth and reproduction occurred resulting in final populations of from 600(07320 worms per tube averaging 3977* i 266 worms for the 32 tubes incubated at 22 degrees C. In sonic cases the animals had just exhausted their food supply at recovery but newly laid eggs were present in the cultures and animals were act ive indicat ing that hermaphrodites were not behaviorl y affected by food

restriction. No obvious pattern in the population size vs incubation condition was detected that was independent of genotype. A slight trend towards larger population size was observed for tubes incubated on centrifuges in space or on the ground. One may speculate that slight vibration induces more movement and feeding of sluggish mutant animals.

Mating

Six fourth larval stage (L4) males were inoculated with six dauer larvae hermaphrodites of either *dpy-11* or *unc-22* genotype and incubated in Incubator A ± centrifuge. The timing of handover and launch assured that virgin males would accumulate sperm for at least one day prior to mating while hermaphrodites developed into young adults. Successful mating would generate outcross heterozygous F_1 animals (50% male) which could engage in a second round of mating to generate Dpy or Unc males in the F_2 generation. Phenotypic ratios of animals present at recovery were measured. New progeny were not counted.

Mating of C. *elegans* is often erratic even under the best of laboratory conditions. One round of mating was efficient in space leading to many outcross progeny and a few F_2 mutant males were also present indicating that a second round of mating between F_1 animals also occurred. Thus the most complex behavior of worms appears to be insignificantly perturbed by microgravity in this hardware configuration. The intrinsic variability prevents a more quantitative assessment.

Segregation

Six triply heterozygous L3 hermaphrodites were inoculated into tubes and incubated in Incubator A ± centrifuge. Developmental timing assures that fertilization could only commence after orbit was achieved and samples were loaded into incubators. The two genotypes were: uric-5 *IVI+;dpy-11   Ihinz-5Vordpy-18IIII +; me-17IV/+; him-5 V/+. The* theoretical Mendelian segregation ratio of 9:3:3:1 should have obtained for Dpy and Unc in the F_1 generation and ¼ of F_1's should also have been *him-5/him-5*. Males would only appear at the spontaneous rate of 1/700 in the F_1. The F_1 Him animals would generate up to 40% males in their broods. Therefore the appearance of Wild Type, Dpy, Unc and Dpy Unc males is due to F_2's. The phenotypic ratios of males present at recovery were measured,

F_1 and F_2 progeny were produced by heterozygous hermaphrodites in proportions which were not significantly different between controls and test samples. The proportions of F_2 males deviated from the theoretical 9:3:3:1 Mendelian ratio but this can be accounted for by different growth rates and fertilities of mutants. As only animals present at recovery were scored (new progeny were ignored) these numbers also represent broods from hermaphrodites whose reproduction was interrupted before completion.

Recombination

Six triply heterozygous L3 hermaphrodites with linked *dpy* and *unc* genes were inoculated into tubes and incubated in Incubator A ± centrifuge. The two genotypes were: *dpy-5 unc-13 I/ + +; him-5 V/+* anti *unc-32 dpy-17 III/ + +; bin?-5 V/ +. The* theoretical Mendelian segregation ratio of 3:1 should have obtained for WT and Dpy Unc in the F_1 generation along with Dpy and Unc recombinants at 1.8 and '2.670 for the two strains, respectively. One fourth of each F, genotype should also have been *him-5/him-5*. As with the "Segregation" test males would be F_2's. Hermaphrodite and male animals present at recovery were scored.

As with segregation, F_1 and F_2 progeny were produced by heterozygous hermaphrodites in proportions which were not significant I y different bet ween controls and test samples. The proportions of F_2 males deviated from the theoretical 3:1 Mendelian ratio, but again this can be accounted for by different growth rates and fertilities of mutants in the mixed generation culture scored at a single time

'period. Many' F_1 recombinant hermaphrodites were seen and a few F_2 males were present.

In typical laboratory experiments it is possible to rigorously separate generations by moving animals regularly to separate petri dishes before they reproduce. However, in the flight experiment, no human intervention occurred for two generat ions, so that the final populations were mixtures of three generations. The growth rates and fertilities of the mutant phenot ypcs are reduced relative to the Wild Type so that systematic distortions to the theoretical Mendelian ratios are expected if animals are scored at any given time point. Thus the most important operational test is a comparison of flight&ground and 1-g & O-g pairs. With these caveats in mind there were no significant differences observed in chromosome mechanics experiments between microgravit y and 1-g.

Anatomical Observations

Several hundred animals were analyzed for their anatomy based on cell number and distribution, nuclear morphology, karyotype and symmetry relations. Particular emphasis was given to the gonad vs intestine symmetry [8] relationship which derives from early (6-cell stage) cell division planes and to the linear gametogenesis sequence of nuclei in the gonad'. No obvious differences have been seen between flight and ground Wild Type animals and no alterations in gonad symmetry were detected with the initial limited sample. No defective karyotypes have been seen and the only unusual feature detected is the presence of intestinal cells with incomplete nuclear divisions in both flight and ground samples.

Observations on a small number of late (at least comma stage) embryos fixed at recovery (about 6 hours after reentry bum restores gravity levels) show no obvious disruptions to anatomy. The timing of development and mission operations is such that post comma stage embryos at recovery were all fertilized and passed through critical developmental events prior to deorbit bum. Young embryos resulted from zygotes which experienced reentry accelerations. In these embryos cellular antomy was also normal and synchronized mitoses were observed suggesting no significant disturbances to cell cycles. The question of maternal spatial patterning in embryos has been addressed using maternal effect embryonic lethal mutations and immunofluorescence of microtubules, actin, P-granules and mambrane antigens. In all cases the evidence points to a homogeneous distribution of cytoplasm in oocytes. The first event establishing polarity is sperm entry at the surface of the oocyte which first enters the spermatheca (valve connecting oviduct and uterus). This site becomes the posterior pole oft he embryo but it is not known whether the anterior-posterior axis exists before sperm entry. Dorso-ventral and left-right axes arc not evident until at least the 4-cell stage[6]. Antibodies directed against germline "P-granules" and the myosin expressed in the pharynx reveal normal staining patterns suggesting normal distribution of embryonic cytoplasmic determinants[6]. P granule segregation has been shown to be controlled by microfila-ment activity in a specific 10 minute interval of the first cell cycle following fertilization. Oniy the P4 cell in young IML- 1 embryos and the Z2 & Z3 germline cells of mature embryos and young larvae contained granules as expected[6]. Pharyngeal myosin was expressed midway through development and its normal structure requires embryonic induction events as well as cell autonomous activit ics at the 6 to 24-ceil stage leading to final development. Figure 5 shows anti- , pharyngeal myosin (D. Miller) anti anti-P-granule antibody (S. Strome) staining in embryos fertilized short] y before the return of gravit y.

Mutagenesis by Space Radiation

Two strategies were used for selection 01 mutations induced by exposure [0 natural space radiation. The first method used a large genetic target of 350 essential genes which are recombination balanced by a reciprocal

Figure S. **Immunofluorescent stain-ing of the head of a larval worm and two embryos which were conceived in microgravity. At left and upper right anti-pharyngeal myosin shows normal distribution in the pharynx and precursor cells reflecting normal segregation of determinants at the 6 blastomere stage. At lower right, anti-P granule staining (green) reveals normal segregation of cyto-plasmic determinants at the lst, 2nd and subsequent divisions in the** P **lineage leading to** germ cells. **Blue counterstaining is from the** DNA specific **dye** DAPL

translocation, *eTl (III; V)*. This method was developed by Rosenbluth and Baillie[5] and has been used to characterize mutagenesis by accelerated chaged particles, neutrons and gamma rays by Nelson and collaborators Cross section vs LET relationships for mutation in mature gametes and dauer larvae gonia have been described and provide a baseline for interpretation of space exposures. The assay measures forward autosomal lethal mutation in regions of chromosomes 3 and 5 corresponding to 15% of the worm genome or 1.2×10^7 base pairs of DNA, Mutants isolated in this way can be classified as to chromosomal location and type including deletion and chromosome duplication. The tester strain, JP1O, was used for these experiments.

The second method utilizes a single large gene, *unc-22*, as a target. Although the target is smaller a strong selection method exists for isolation of *unc-22* alleles and their "twitching" phenotypes are not found associated with mutations at any other locus. The real advantage of this method is the availability of molecular probes for DNA hybridization characterization of mutants and the extensive characterization of the chromosome region around the gene. These features are described in reference 4. A variety of *unc-22* mutants induced by accelerated particles once again serves as a baseline for comparison of IML- 1 mutants and their structural spectrum varies with particle properties The wild type strain, N2, was used for *unc-22* mutagenesis. JP1O and N2 animals were inoculated into both immobilized stacks and tube cultures for isolation of lethals and twitcher mutants.

Mutant isolation required a very large logistics effort to prepare and handle over 70,000 cultures for initial screening and resulted in lethal and *unc-22* mutants in each t ypc of hardware component. Mutants have been characterized by Southern hybridization methods and shown to contain deletions as large as 13 kb.Preliminary yields arc: 12 *unc-22* mutants from 1.1 million F_1 animals exposed in the Spacelab tunnel, 1 uric-22 from 188,000 F_1's derived from immobilized dauers and 2 spontaneous mutants from 3.8 million ground control F_1's which matches the laboratory spontaneous rate.

Approximately 60 lethal mutants were isolated from flight samples and anothcr6 in ground controls at a rate which again matches laboratory spontaneous rates. The frequency of flight mutants was about eight-fold above background supporting the conclusion that they were. in fact, radiation-induced. An initial classification scheme based on segregation ratios of offspring and fertility suggests that the spectra of mutants isolated randomly from tubes is qualitatively different from those correlated with specific tracks (Figure 7). Abnormal ratios and low fertility arc often associated with chromosomal rearrangements. If this trend holds for I ML-1 mutants after genct ic characterization then the specific track mutants should show a higher frequency of rearrangements and deletions than tube mutants. The ray-tracing method enhanced the "capture" efficiency over random screening several fold.

Figure 6. Distribution of locations of high LET cosmic ray tracks from which JP1O animals were extracted for mutant isolation. The location from which mutant FP32 was isolated is indicated by a square. The scale lines are in mm.
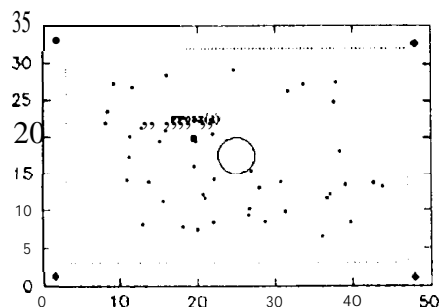
. Physical dosimetry for each location was performed by developing TLDs and nuclear track detectors. The total TLD dose varied from approximately 0.8 mGy in Biorack locations to 1.1 mGy in the Spacelab tunnel. Integral LET spectra from CR-39 detectors showed a typical cosmic ray distribution expected for a high inclination orbit. Specifically the spectra agree well with those obtained for STS-27 and STS-28. The spectra also showed an enhancement of fluence in the Spacelab tunnel over the more heavily shielded Biorack.
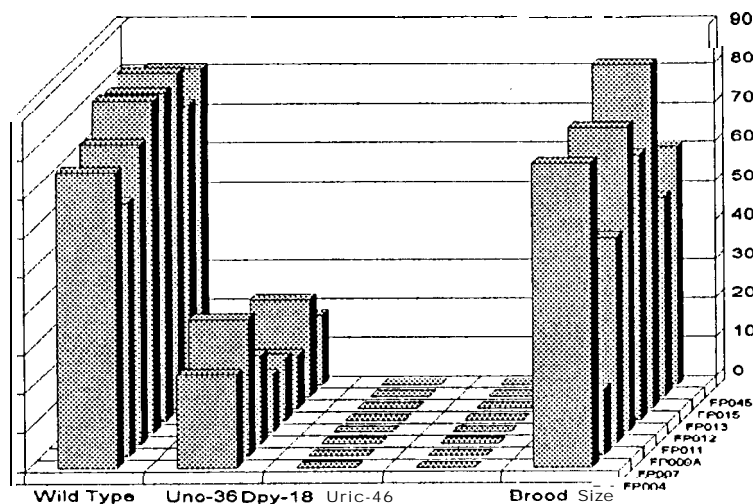
Figure 7. An analysis of phenotypes of 16 lethal mutants isolated from sandwich assemblies shows that both mutants of ideal phenotypic ratio and fertility (panel A) and those of altered ratios (panel B) were isolated. The latter category is characteristic of chromosomal rearrangements. Mutants isolated using the ray tracing method were predominantly of the altered form whereas those from tubes were of the ideal type. This finding is consistent with the interpretation that higher LET particles favor larger scale genetic lesions. Genetic characterization of these mutants is in progress.

7A.

711.

Summary
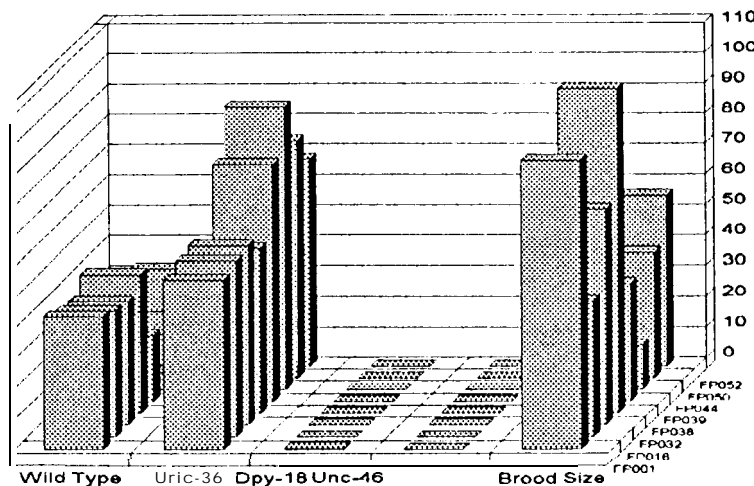
Based on preliminary observations there arc no obvious differences in the development, behavior and chromosome mechanics of *C.elegans* as a function of gravity unloading. The animals successfully reproduced twice in space with ' the generation of many thousands of offspring. Both self-fertilization and mating of males with hermaphrodites was successful. Gross anatomy, symmetry and gametogenesis were normal based on light microscope observations. No defective karyotypes or cell distributions were observed. Finally, the pairing, disjoining and recombination of chromosomes showed no differences correlated with gravity levels. The total doses to samples of 0.80 to 1.10 mGy were not expected to perturb development in any significant way.

9

A variety of mutants were isolated in the unc-22 gene and in essential genes balanced by the eTl translocation. Phenotypic assessment of mutants suggests that mutants isolated from regions of identified high LET particles are more severe than those isolated by random screening. Large deletions have been identified amongst the uric-22 mutants. The rates of mutagenesis were significantly above those in ground controls. Genetic characterization of mutants is continuing.

Physical dosimetry shows that the dose and particle spectrum measured on IML-1 is similar to that seen on other high inclination space shuttle flights. The frequency of mutant isolation was found to correlate with total energy deposited in the immediate vicinity of worms.

References

1. Hirsh, D, Oppenheim, D, Klass, M, Development of the reproductive system of *Caenorhabditis elegans*. *Develop. Biol.*, *49,* pp. 200-219 (1976)

2. Nelson, G A, Schubert, W W, Marshall, TM. Radiobiological studies with the nematode *Caenorhabditis elegans*. Genetic and developmental effects of high LET radiation. *Nucl. Tracks Radiat. Meas.*, *20,* pp. 227-232 (1992)

3. Nelson, G A, Schubert, W W, Marshall, T M, Benton, E R, Benton, E V, Radiation effects in *Caenorhabditis elegans*. Mutagenesis by high and low LET ionizing radiation. *Mutation Res.*, *212.* pp. 181-192 (1989)

4. Rogalski, T M, Baillie, D L, Organization of the unc-22 IV gene and the adjacent region in *Caenorhabditis elegans*. *Mol. Gen. Genet.*, *201, pp. 409-414 (1985)*

5. Rosenbluth, R E, Cuddeford, C, Baillie, D L, Mutagenesis in *Caenorhabditis elegans*. II. A spectrum of mutational events induced with 1500 rad of gamma-radiation. *Genetics*, 109, pp. 493-511 (1985)

6. Strome, S, Generation of cell diversity during early embryogenesis in the nematode *Caenorhabditis elegans. Int. Rev. Cytol.*, **114,** pp. 81-123 (1989)

7. Strome, S, Wood, W B, Generation of asymmetry and segregation of germ-line granules in car] y C. *elegans* embryos. *Cell*, 35, pp. 15-25 (1983)

8. Wood, W B, Evidence from reversal of handedness in C. *elegans* embryos for early cell interactions determining cell fates. *Nature*, *349,* pp. *536-538 (1991)*

9. Wood, W B. editor, The nematode *Caenorhabditis elegans*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., (1988)